A pool of β-tubulin is hyperphosphorylated at serine residues in Alzheimer disease brain

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Abstract In Alzheimer disease (AD) brain, activities of protein phosphatase (PP)-2A/PP-1 which are known to be associated with microtubules are compromised and are probably a cause of neurofibrillary degeneration through hyperphosphorylation of microtubule proteins. In the present study, an increase of ~11 pmol phosphate/μg protein in 100 000×*g* pellet from AD compared with age-matched control brains was found. Tau protein, which is hyperphosphorylated in AD can only account for ~4 pmol phosphate/μg protein, suggesting the presence of non-tau hyperphosphorylated proteins in the diseased brain. Western blot analysis with phosphoserine antibodies revealed a ~54 kDa non-tau protein to be significantly hyperphosphorylated in AD compared with age-matched control cases in the particulate fraction. The ~54 kDa protein was purified by preparative sodium dodecyl sulfate–polyacrylamide gel electrophoresis and identified as β-tubulin by immunolabeling with specific antibodies, mass spectrometry analysis and by N-terminal amino acid sequencing. The purified protein was hyperphosphorylated at serine residues in AD. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Alzheimer disease; Hyperphosphorylation; β-Tubulin; Phosphoserine

1. Introduction

In Alzheimer disease (AD) brain the neuronal cytoskeleton is disrupted and replaced by the accumulation of paired helical filaments (PHF). Microtubule (MT)-associated protein tau, the major protein subunit of PHF, is abnormally hyperphosphorylated and an imbalance in the protein kinase-protein phosphatase (PP) system has been implicated in this abnormal phosphorylation [1–5]. Furthermore, the activities of phosphoseryl/phosphothreonyl PP-1 and -2A, which are associated with MTs [6,7], expressed in neurons [8] and regulate the phosphorylation of neuronal proteins at serines and threonines [9–12], are decreased in AD brain [13,14]. Employing metabolically active rat brain slices as a model system and inhibition of PP-2A by okadaic acid, Bennecib et al. [15] have demonstrated that a decrease in PP-2A activity leads to the hyperphosphorylation of tau not only by a decrease in its dephosphorylation but also by an increase in calcium-calmodulin-dependent protein kinase II activity which is regulated by this phosphatase. A recent study has reported an increase in cyclin-dependent protein kinase-5 activity in AD brain mediated by the conversion of a rather unstable regulatory subunit p35 to a relatively more stable p25, which accumulates and activates this kinase [17]. However, Taniguchi et al. [18] have been unable to reproduce these findings. The abnormal hyperphosphorylation of tau and changes in enzyme activities that regulate the intracellular protein phosphorylation/dephosphorylation are consistent with the hypothesis that there is a general imbalance in the protein phosphorylation/dephosphorylation system and that in addition to tau one or more phosphoproteins in the affected neurons might be abnormally hyperphosphorylated in AD brain.

MTs, which are required for axonal transport and thus are critical to neuronal function, are rarely seen in neurons with neurofibrillary tangles [19]. The major MT protein subunit, tubulin, is a 100–110 kDa heterodimer of α- and β-tubulin [20]. Both of these tubulins exist in several isotypic forms, encoded by different genes [21]. Of the various tubulin isotypes, β-III isotype was previously shown to be phosphorylated [22–24]. Although phosphorylation of β-III-tubulin does not affect its polymerization into MTs, it inhibits the microtubule-associated protein (MAP)2-promoted MT assembly, suggesting that this post-translational modification might be involved in the regulation of MT assembly through tubulin–MAP interaction [25].

Here, we report the hyperphosphorylation of a 54 kDa non-tau phosphoprotein in the 100 000×*g* particulate fraction of brain of AD patients. This ~54 kDa protein, which was found to be hyperphosphorylated at serine residues in AD compared with age-matched controls, was purified by preparative sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and identified as β-tubulin by immunolabeling with specific antibodies to β-tubulin, by mass spectrometry (MS) and by N-terminal amino acid sequencing.

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Abbreviations: AD, Alzheimer disease; PP, protein phosphatase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PHF, paired helical filament; MES, 2-[*N*-morpholino]ethanesulfonic acid; IMAC, iron metal affinity chromatography; TBST, Tris-buffered saline with Tween 20; MS-MALDI, mass spectrometry-matrix assisted laser desorption/ionization

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2. Materials and methods

2.1. Brain tissue

The brains of 15 AD patients with a mean age of 71.8 years (±8.3 years) were employed for the present study. All the AD brains had been histopathologically confirmed and contained the hallmark lesions specific for AD, i.e. plaques and tangles. Post-mortem delays were 4.0±1.5 h. The mean age of the 13 non-AD brains used as controls in this study was 79.4 years (±4.8 years) and the post-mortem delays were 4.5±1.0 h. The human tissues were obtained from the Brain Tissue Resource Center, McLean Hospital, Belmont, MA, USA and The Netherlands Brain Bank (Dr. Rivka Ravid), The Netherlands, and from our Institute for Basic Research Brain Bank (Dr. Peter Koslowski), Staten Island, NY, USA.

2.2. Removal of non-protein phosphates

Neocortices from AD or control brains, cleaned free of meninges and underlying white matter were homogenized at 4°C in 0.1 M 2-[*N*-morpholino]ethanesulfonic acid (MES) buffer, pH 5.7, 1 mM dithiothreitol (DTT), 50 mM NaF, 1 mM Na_3VO_4 and 1 mM phenylmethylsulfonyl fluoride (PMSF) (1:9 w/v) using a Potter–Elvejam homogenizer. The homogenates were centrifuged at 100 000×*g* for 30 min at 4°C to obtain the supernatant and the pellet fractions; non-phosphoprotein phosphate was removed as described previously [26,27]. Briefly, to an aliquot of each sample, 0.0125% Na-deoxycholate and 6% trichloracetic acid (TCA) were added to precipitate proteins. After centrifugation in a Beckman Model GS-6R centrifuge at 2600×*g* for 30 min, ether:ethanol (1:1) mixture was added to the pellet and mixed thoroughly before centrifugation. The pellet obtained was then boiled in 16% TCA for 20 min and centrifuged as above. The pellets were washed with 10% TCA before resuspending in 0.1 N NaOH. Protein was assayed by modified Lowry's method as described by Bensadoun and Weinstein [28] and required amounts of protein were dried in thick-walled glass tubes using a Jouan RC 10.22 vacuum concentrator, after which the dried samples were used for phosphate analysis.

2.3. Phosphate analysis

For total phosphate estimation, to the dry samples 60 μl of 5% $MgNO_3$ in 95% ethanol was added. The samples were heated on a heating block for 20 min, followed by ashing over open flame, until ash was formed. The ash was dissolved in 40 μl of 1 N H_2SO_4, followed by centrifugation to get rid of any precipitates that may interfere with the assay. Then, in a microtiter plate, 25 μl of the supernatant/well was transferred and mixed with 50 μl of dye mixture (0.045% malachite green, 4.2% ammonium molybdate in 4 N HCl, 3:1 mixed with 100 μl of 2% Tween 20). After 15 min incubation, optical density was read at 660 nm. Non-ashed protein samples treated as above except the ashing step were employed to determine the levels of any free phosphate. The values for protein-bound phosphate were calculated by subtracting the values of inorganic/free phosphate from the total (ashed samples) phosphate [26].

2.4. Separation of phosphoproteins by iron metal affinity chromatography (IMAC)

Chelating Sepharose was packed in a column (1×4 cm; 2 ml bed volume) and 2.5 bed volumes of 50 mM $FeCl_3$ was passed through the column. This was followed by extensive washing of the column with distilled water, until the eluent became colorless. The column was then equilibrated with 0.1 M MES, pH 5.7, containing 1 mM DTT, 50 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF. After loading the 100 000×*g* brain extract (see Section 2.2) on the column, the proteins were eluted by step elution, first by using 0.1 M 3-[*N*-morpholino]propanesulfonic acid, pH 7.0, containing 1 mM DTT, 50 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF and 1 M NaCl and then by 0.1 M Tris, pH 9.0, containing 1 mM DTT, 50 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF and 1 M NaCl. The flow rate was 15 ml/h throughout the procedure [29].

2.5. Western blots

The 100 000×*g* pellet or supernatant of the tissue homogenate was subjected to electrophoresis on a 5–15% gradient SDS–PAGE [30]. Proteins from the gel were transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% BSA–TBST (bovine serum albumin–50 mM Tris buffer, pH 7.6, 0.9% NaCl, 0.05% Tween 20) and incubated with the appropriate primary antibody, followed by incubation with ^{125}I-labeled or horseradish peroxidase (HRP)-labeled secondary antibody. The membranes were washed adequately with TBST before exposing to a phosphorimager screen or developing with enhanced chemoluminescence (ECL) reagents (1:1), followed by exposure to Kodak X-ray film. The immunoreactivity was quantitated using a phosphorimager (Fuji film BAS-1500, Japan). ^{125}I-labeled anti-mouse and anti-rabbit secondary antibodies were purchased from Amersham, Piscataway, NJ, USA. Anti-mouse and anti-rabbit HRP-labeled secondary antibodies were from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Polyclonal anti-phosphoserine (P-Ser) antibodies were from Zymed Labs, San Francisco, CA, USA, and monoclonal anti-β-tubulin was from Calbiochem, San Diego, CA, USA.

2.6. Purification of the ∼54 kDa protein by preparative SDS–PAGE

The 100 000×*g* pellet of brain homogenate was first extracted with a mixture of detergents (1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) by incubating for 3 h at room temperature (RT) with intermittent mixing, followed by centrifugation at 100 000×*g* for 30 min at 4°C. The detergent insoluble fraction was incubated in 6 M guanidinium hydrochloride (GnHCl) for 3 h at RT with intermittent mixing, followed by centrifugation as above. The GnHCl soluble fraction was dialyzed against 2 M urea containing 50 mM Tris, pH 7.6, 25 mM NaF, 1 mM Na_3VO_4, 1 mM DTT, and ∼1 mg protein was loaded on a 10% preparative SDS–PAGE (Model 491 Prep Cell from Bio-Rad, CA, USA) and 288 fractions were collected at an elution rate of 60 ml/h. Fractions containing the ∼54 kDa protein were detected by monitoring all fractions by both Coomassie blue staining as well as Western blot analyses with P-Ser antibodies (Zymed Labs, San Francisco, CA, USA).

2.7. MS of the purified protein

The Coomassie blue-stained protein was excised from a 10% SDS–PAGE gel, washed 25 times with distilled water, to minimize SDS content, and dried using a vacuum concentrator. The dried gel was hydrated in 100 μl of 100 mM Tris, pH 8.8, followed by addition of 10 μl of 45 mM DTT and incubation for 30 min at 55°C. The solution was discarded and the gel was taken up in 100 μl of 100 mM Tris, pH 8.8, 10 μl of 100 mM iodoacetamide, and incubated for 1 h in the dark at RT. The gel was then washed with 500 μl of 50 mM Tris, pH 8.8/50% acetonitrile (ACN) for 30 min, and gel-dried to completion in a vacuum concentrator. The dried gel containing the protein was then rehydrated in 40 μl of 25 mM Tris, pH 7.8, and the protein in the gel was digested with 1 pmol of LysC (sufficient volume to cover the gel) overnight at 37°C. Peptides were extracted from the gel with 50% ACN/0.1% trifluoroacetic acid (TFA) for 30 min at 37°C. The extract was removed from the gel and vacuum-concentrated to about 10 μl. Then, 2 μl digested peptides were mixed with 1 μl of matrix solution (10 mg/ml of α-cyano-4-hydroxycinnamic acid in 0.1% TFA/33% ACN/33% ethanol) and spotted on a matrix-assisted laser desorption/ionization (MALDI) plate in aliquots of about 1 μl. MS-MALDI was performed on a Perkin-Elmer Voyager DE-RP machine using reflector mode to obtain monoisotopic peptide masses.

2.8. N-terminal amino acid sequencing of the purified ∼54 kDa protein

The Coomassie blue-stained purified protein was excised from a 10% SDS–PAGE, washed and vacuum-dried as described for MS analysis. Automated Edman sequencing for the purified ∼54 kDa protein was performed using a Procise 494 ABI sequenator with reagents and methods of the manufacturer.

3. Results

3.1. Phosphoprotein phosphate levels in the frontal gray matter of AD and age-matched control cases

In order to determine the general phosphorylation status of brain proteins, the 100 000×*g* pellet, 100 000×*g* supernatant and the IMAC fractions of 100 000×*g* supernatant – unbound, pH 7.0 and pH 9.0 elutes of five AD and five age-matched control cases were assayed for protein-bound phosphate as described in Section 2. The 100 000×*g* pellet of AD cases contained ∼11 pmol phosphate/μg protein more than the age-matched controls (Table 1), whereas the 100 000×*g*

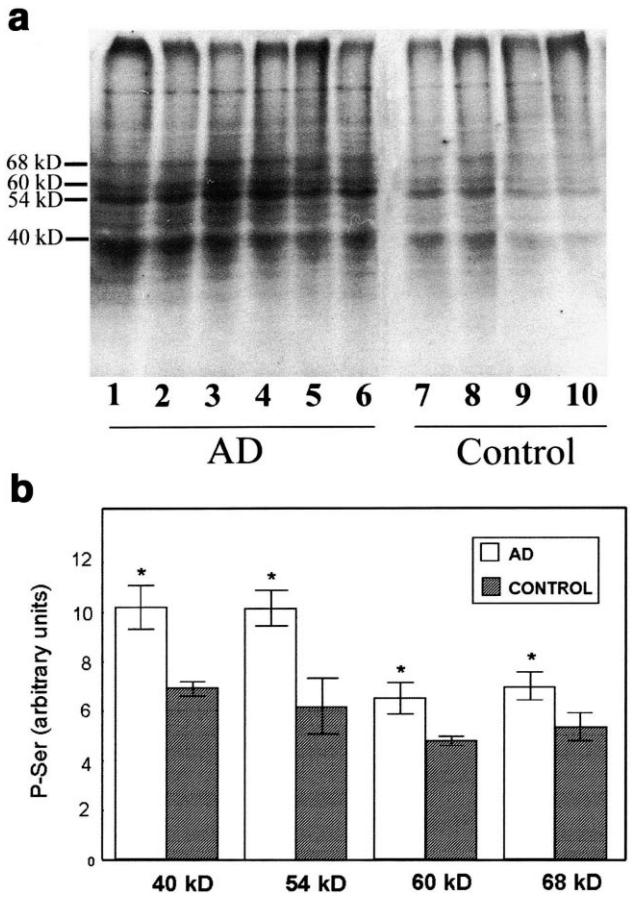


Fig. 1. Western blots of the 100 000×*g* pellet of frontal gray matter homogenates from AD and age-matched control cases developed with antibody to P-Ser. a: The 100 000×*g* pellets of gray matter homogenates from AD and age-matched control cases were electrophoresed (30 μg/lane) on 5–15% SDS–PAGE and the proteins transferred to polyvinylidene difluoride membrane. After blocking with 5% BSA–TBST, the phosphorylated serine residues of proteins were immunodetected using polyclonal P-Ser antibodies (1:750) and HRP-conjugated secondary antibody (1:25 000). The blots were developed by ECL reagents. Lanes 1–6: AD; lanes 7–10: age-matched controls. The intensity of immunoreactivity was quantitated by densitometry. b: P-Ser immunoreactivity was significantly higher in ~40, ~54, ~60 and 68 kDa polypeptides in AD compared with age-matched controls.

supernatant and the three IMAC fractions from the supernatant did not reveal any statistically significant differences between AD and age-matched control cases (Table 1).

3.2. Identification of a 54 kDa protein hyperphosphorylated in AD brain

In order to identify hyperphosphorylated proteins, the 100 000×*g* pellet, which showed increased levels of phosphoprotein phosphate in AD cases, was analyzed by Western blots developed with antibodies that specifically recognized P-Ser, phosphothreonine or phosphotyrosine residues. At least four polypeptides were found to be hyperphosphorylated at serine residues in the AD frontal gray matter as compared with the corresponding tissue from age-matched controls (Fig. 1a,b). A ~54 kDa protein was ~50% more hyperphosphorylated in AD than control brains.

While three of the four hyperphosphorylated polypeptides were also tau immunoreactive, the ~54 kDa hyperphosphorylated protein was found to be tau immuno-negative (data not shown). The identity of the ~54 kDa hyperphosphorylated protein was further pursued. No changes in phosphorylation at serine residues were observed in the 100 000×*g* supernatant of AD cases compared with age-matched controls (data not shown).

Table 1
Picomol of phosphate per microgram of protein in the supernatant, the three IMAC fractions and the 100 000×*g* pellet of AD and control gray matter homogenates.

Fraction	AD	Control
Supernatant	10.0 ± 0.8	10.4 ± 1.9
Unbound	9.9 ± 5.0	10.3 ± 4.4
pH 7.0 eluate	6.3 ± 1.4	6.7 ± 1.4
pH 9.0 eluate	19.8 ± 6.8	22.5 ± 5.8
Pellet	75.5 ± 4.7	63.9 ± 5.4

3.3. Solubilization of the ~54 kDa protein

A battery of detergents/denaturants were utilized to solubilize the 100 000×*g* pellet proteins. The hyperphosphorylated ~54 kDa non-tau protein was insoluble in 1% Triton X-100, 6% Triton X-100, 1% NP-40 and in a mixture of detergents

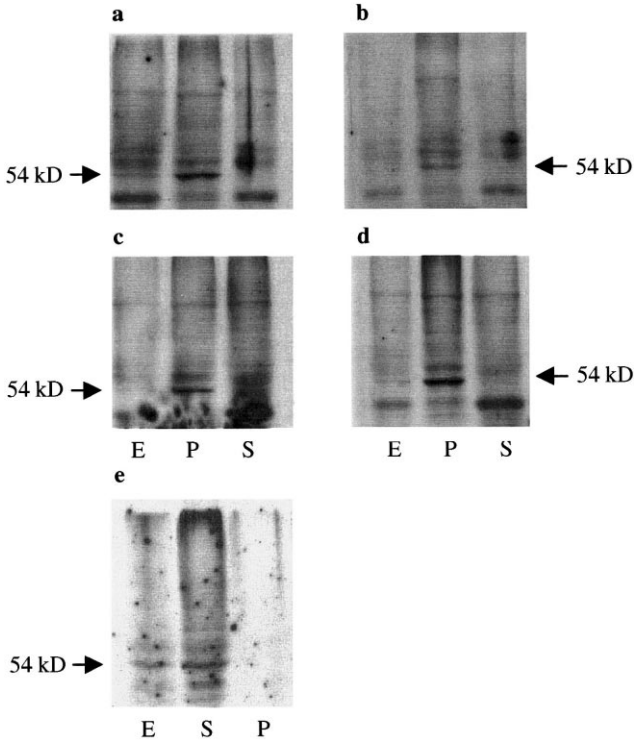
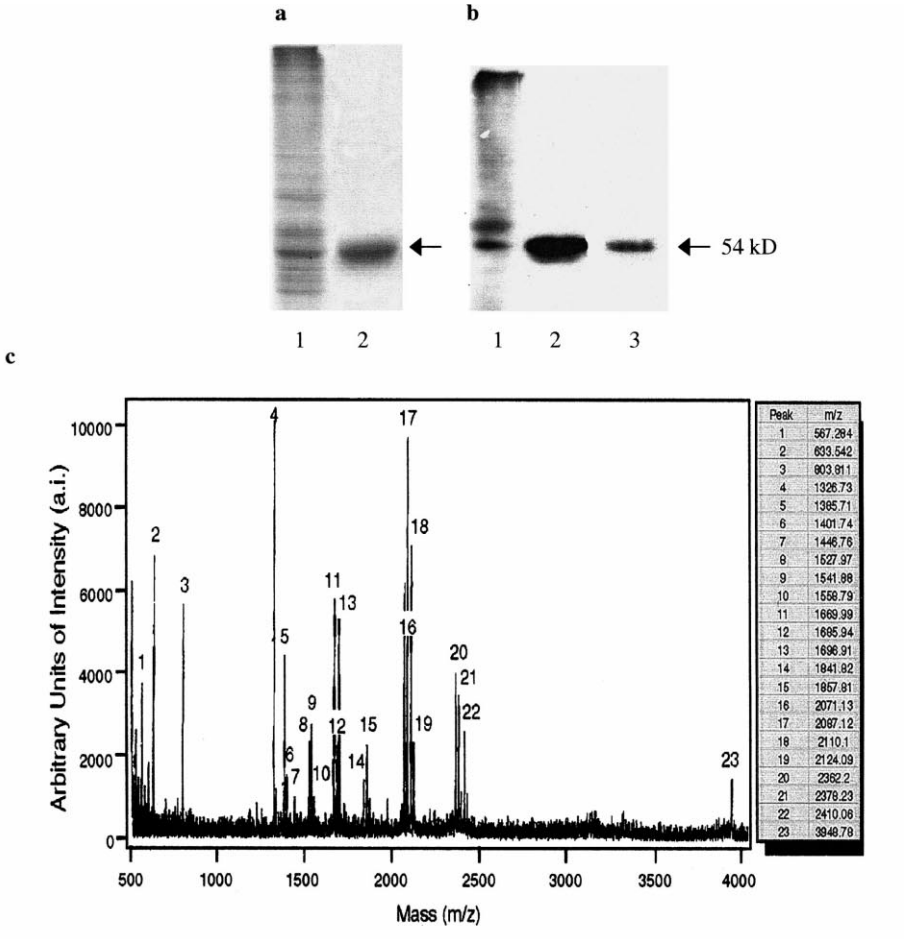


Fig. 2. Extraction of the ~54 kDa protein from the 100 000×*g* pellet of brain homogenate. Results of extractions by detergents and GnHCl as analyzed by SDS–PAGE and Western blots. Six milligrams of 100 000×*g* pellet protein was incubated individually with 3 ml of (a) 1% Triton X-100, (b) 6% Triton X-100, (c) 1% NP-40, (d) 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS or (e) 6 M GnHCl for 3 h at RT with intermittent mixing before centrifugation at 100 000×*g* for 30 min at 4°C. The supernatant (S) was considered the detergent/denaturant soluble pool of protein and the pellet (P), the insoluble pool. Sample before centrifugation was termed extract (E). All the different pools of protein were then analyzed for the presence of a ~54 kDa protein by Western blots developed with P-Ser antibodies (1:750). The ~54 kDa protein remained insoluble in all the detergents used (a–d) and was soluble only in 6 M guanidine hydrochloride (e).



d

Alzheimer disease			Control		
Residues	*Amino Acids*	*Yield (pmoles)*	*Residues*	*Amino Acids*	*Yield (pmoles)*
1	M	3	1	M	3
2	R	6	2	R	5
3	E	3	3	E	3
4	I	3	4	I	3
5	V	3	5	V	3
6	H	2	6	H	1
7	I	2	7	I	2
8	Q	1	8	Q	2
9	A	2	9	A	2
10	G	1	10	G	1

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>gi|631483|pir||S45140 tubulin beta chain - human
MREIVHIQAGQCGNQIGAKFWEVISDEHGIDPTGSYHGDSDLQLERINVYYNEAAGNKYVPRAILVDLEP
GTMDSVRSGPFGQIFRPDNFVFGQSGAGNNWAKGHYTEGAELVDSVLDVVRKESESCDCLQGFQLTHSLG
GGTGSGMGTLLISKIREEYPDRIMNTFSVMPSPKVSDTVVEPYNATLSVHQLVENTDETYSIDNEALYDI
CFRTLKLTTPTYGDLNHLVSATMSGVTTCLRFPGQLNADLRKLAVNMVPFPRLHFFMPGFAPLTSRGSQQ
YRALTVPELTQQMFDSKNMMAACDPRHGRYLTVAAIFRGRMSMKEVDEQMLNVQNKNSSYFVEWIPNNVK
TAVCDIPPRGLKMSATFIGNSTAIQELFKRISEQFTAMFRRKAFLHWYTGEGMDEMEFTEAESNMNDLVS
EYQQYQDATADEQGEFEEEEGEDEA
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containing 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS (Fig. 2a–d), whereas treatment of the 100 000×*g* pellet with 6 M GnHCl solubilized the ~54 kDa protein (Fig. 2e). About 60% of the 100 000×*g* pellet protein was soluble in the mixture of detergents. Further extraction of the detergent insoluble pellet with 6 M GnHCl yielded an enrichment of the ~54 kDa protein. The GnHCl extract which was enriched in the ~54 kDa hyperphosphorylated protein was dialyzed against Tris buffer containing 2 M urea before subjecting to purification by preparative SDS–PAGE.

3.4. Purification of the ~54 kDa protein by preparative SDS–PAGE

The ~54 kDa protein-enriched preparation in 2 M urea was subjected to 10% preparative SDS–PAGE to isolate the ~54 kDa hyperphosphorylated protein. Only two to three fractions matched both the correct molecular size, as monitored by Coomassie staining, and the intense P-Ser immunoreactivity pattern (note that the ~54 kDa protein is a predominant P-Ser immunoreactive band in Fig. 1 as well as Fig. 3b, lane 1), as determined by Western blots with P-Ser anti-

Fig. 3. Purification of the ~54 kDa protein and its identification as β-tubulin. The 100 000×*g* pellet from AD brains was subjected to detergent (1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) extraction, followed by the extraction of the insoluble pellet with 6 M GnHCl. The 6 M GnHCl extract was dialyzed against 50 mM Tris, pH 7.6, 2 M urea, 1 mM DTT, 50 mM NaF, 1 mM Na_3VO_4 and 1 mM PMSF, and 1 mg of dialyzed protein was subjected to preparative SDS–PAGE. Protein fractions were collected and the ~54 kDa protein-containing fraction was identified on the basis of screening all the fractions by both Coomassie blue staining and Western blotting with P-Ser antibodies. a: Coomassie blue staining of protein on a 10% SDS–PAGE. Lane 1 – GnHCl extract; lane 2 – purified ~54 kDa protein-containing fraction. b: Western blot with P-Ser antibody (1:750) or β-tubulin antibody (1:750). Lane 1 – GnHCl extract; lanes 2,3 – purified ~54 kDa protein-containing fraction from SDS–PAGE developed with antibodies to P-Ser (lane 2) or to β-tubulin (lane 3). c: MS-MALDI spectrum of Lys-C peptides obtained from the purified ~54 kDa protein. A total of 23 mass/charge (*m/z*) peaks were obtained. Upon matching with the data bank, the ~54 kDa protein was identified as β-tubulin. Abscissa: *m/z* ratio of the peptide fragments. Ordinate: Arbitrary units of intensity (a.i). The table (right) lists the mass signals (1–23) attributed to Lys-C fragments of the ~54 kDa protein with measured *m/z* values. d: N-terminal amino acid sequence analysis of the ~54 kDa protein purified by 10% preparative SDS–PAGE. The Coomassie-stained protein band was excised from the gel and subjected to amino acid sequence analysis. The N-terminal sequence MREIVHIQAG obtained by this analysis (upper panel) matched with that of β-tubulin (lower panel), confirming the identity. The results also revealed that the ~54 kDa protein was probably pure. Lower panel – the sequence of β-tubulin that identified with the ~54 kDa protein by MS-MALDI analysis.
←

bodies (Fig. 3a–b). The ~54 kDa hyperphosphorylated protein was excised from Coomassie blue-stained gel (Fig. 3a) for identification by MS.

3.5. *Identification of the ~54 kDa hyperphosphorylated protein as β-tubulin*

The purified ~54 kDa protein excised from Coomassie blue-stained gel was digested with Lys-C before subjecting to MS-MALDI. The spectrometric analysis revealed the identification of the ~54 kDa protein as β-tubulin (accession number X79535; Fig. 3c). The identification of the ~54 kDa protein as β-tubulin was confirmed by Western blots developed with antibodies to β-tubulin (Fig. 3b, lane 3). Amino-terminal amino acid analysis (carried out at a sensitivity of 0.5 pmol for each amino acid residue) of the ~54 kDa protein revealed a single polypeptide which matched with the previously known amino acid sequence of β-tubulin identified by MS-MALDI (Fig. 3d).

3.6. *Preparative electrophoresis-purified β-tubulin is hyperphosphorylated at serine residues*

In order to confirm whether β-tubulin from AD brain was indeed hyperphosphorylated as compared with that of age-matched control brains, the preparative SDS–PAGE-purified ~54 kDa protein, identified as β-tubulin, was subjected to Western blots developed with antibodies to P-Ser. The P-Ser immunoreactivity normalized against β-tubulin immunoreactivity was found to be 43% more in purified β-tubulin from AD compared with age-matched controls, confirming that this protein is hyperphosphorylated at serine residues in AD brain (Fig. 4).

4. Discussion

Previous studies in which tau was found to be abnormally hyperphosphorylated had hypothesized that there might be a protein phosphorylation/dephosphorylation imbalance in AD brain [1–4]. In the present study, we have found that the particulate pool of β-tubulin, a non-tau protein, is also abnormally hyperphosphorylated in AD brains.

The level of the abnormally hyperphosphorylated tau (for an average molecular weight of 45 000) is ~0.2–0.4 pmol/μg 100 000×*g* pellet protein from AD brain [31,32], and it has a phosphorylation stoichiometry of ~6–10 mol phosphate/mol of the protein [27]. In the present study, we found that the 100 000×*g* pellet of gray matter homogenates from AD cases had an increase of ~11.0 pmol phosphate/μg protein. These data indicated the presence of other abnormally hyperphosphorylated proteins in AD brain. Western blot analyses with P-Ser antibodies revealed hyperphosphorylation of several proteins. All detectable proteins, except a ~54 kDa protein, were also labeled with antibodies to tau, indicating hyperphosphorylation of a non-tau protein. We purified the ~54 kDa protein by preparative SDS–PAGE. MS-MALDI analy-

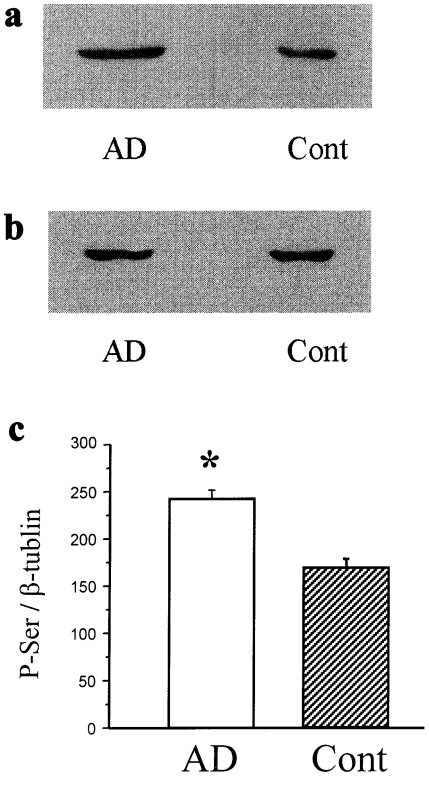

Fig. 4. Western blots of the preparative SDS–PAGE-purified ~54 kDa protein developed with antibody to P-Ser. Preparative electrophoresis-purified ~54 kDa protein from AD brain and from age-matched control brains was electrophoresed on a 10% SDS–PAGE and transferred to a polyvinylidene difluoride membrane. After blocking with 5% BSA–TBST, (a) phosphorylated serine residues were immunodetected using polyclonal antibodies to P-Ser (1:750) and (b) β-tubulin levels were immunodetected using monoclonal β-tubulin antibody (1:750) followed by corresponding HRP-conjugated secondary antibody (1:25 000). P-Ser immunoreactivity normalized against that of β-tubulin from blots as in (a) and (b) was significantly ($P < 0.05$) higher in AD than in control brains (c). The blots were developed by ECL reagents. The data in (c) is from three AD and three control values.

sis revealed the identity of the ~54 kDa protein as β-tubulin (X79535). We confirmed the identity of the purified protein by Western blots developed with anti-β-tubulin antibodies, and by amino-terminal amino acid sequencing. Amino-terminal sequencing revealed that not only was the protein β-tubulin, but also probably was pure. Western blots of the purified protein developed with P-Ser and β-tubulin antibodies confirmed that β-tubulin from AD brains was indeed hyperphosphorylated as compared with that from control brains. Hyperphosphorylation of β-tubulin, a protein other than tau, in AD brain is consistent with the hypothesis that a general imbalance in the protein phosphorylation–dephosphorylation system exists in the brains of AD patients. Other proteins which have been shown to be hyperphosphorylated at serine/threonine residues in AD brain include dynein [33], CRMP-2 [34] and neurofilament heavy and medium subunits [35].

Tau is required for MT assembly, maintenance of MTs and stability of MTs [36]. A large body of evidence has established that hyperphosphorylated tau contributes to the MT disassembly. It has been demonstrated that unlike normal tau, AD P-tau cannot promote MT assembly, cannot stabilize MT or maintain the MT structure [37–39]. AD P-tau sequesters normal tau, MAP1 and MAP2, and makes them unavailable for MT assembly [40]. But, the possible role of tubulin in AD brain towards the disassembly of MTs has not been considered previously. A study has shown that bovine brain tubulin when phosphorylated by a serine-threonine kinase, calcium-calmodulin protein kinase, loses its ability to assemble MTs and when dephosphorylated, tubulin regains its ability to assemble MTs [41]. The activity of phosphoseryl/phosphothreonyl PP-2A is decreased in AD brain [13,14] and an inhibition of PP-2A activity in brain has been shown to result in an increase in the activity of calcium-calmodulin-dependent protein kinase II [15]. Phosphorylation has also been shown to enhance the interaction of tubulin with the membrane [42]. Thus, hyperphosphorylation of β-tubulin in the $100\,000 \times g$ particulate fraction might have resulted from the decrease in PP-2A activity in AD brain.

The hyperphosphorylated β-tubulin observed by us might be either from the membrane pool or cytoplasmic pool. If it is from the cytoplasmic pool, which represent over 90% of total brain tubulin, then only a small fraction of it might have aggregated as a result of hyperphosphorylation into the particulate pool. β-Tubulin (X79535) is not a major isotype of β-tubulin in neurons. β-III-Tubulin, which is the predominant isotype in neurons, has been shown to be phosphorylated, whereas type II and type IV were not found to be phosphorylated [25]. Therefore, a slight decrease in the specific isotype (X79535) in the $100\,000 \times g$ supernatant may remain undetected considering its relatively small contribution to this pool. Although the contribution of β-tubulin (X79535) in MT assembly in a young, healthy brain may not be very significant, it might play a significant role in the AD brain for the following reasons. In the brains of AD patients, normal tau is sequestered by AD P-tau which might result in a decrease in available normal tau for MT assembly. Under these conditions, even a small amount of hyperphosphorylated β-tubulin (presumably in a compromised functional state) might contribute significantly to the disassembly of MTs either directly or through tubulin–MAP interaction proposed previously [25]. If the hyperphosphorylated β-tubulin (X79535) belonged to the membrane pool, it would be hard to attribute any function, since this area of research remains unexplored.

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